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Award Number: DAMD17-02-1-0583

TITLE: The Role of Ubiquitin-Mediated Proteolysis of Cyclin D in Breast Cancer

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REPORT DATE: April 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2004	Annual Summary (1 Apr 2003 - 31 Mar 2004)

4. TITLE AND SUBTITLE

The Role of Ubiquitin-Mediated Proteolysis of Cyclin D in Breast Cancer

5. FUNDING NUMBERS

DAMD17-02-1-0583

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Cyclin D is a positive regulator of the cell cycle and is found to be highly expressed in breast cancer cells. Cyclin D is post-transcriptionally regulated by the ubiquitin mediated protein degradation pathway. CDC34, a ubiquitin conjugating enzyme, and SCF (Skp1, Cullin, F-box, ring protein), a ubiquitin ligase, are postulated to be the specific E2 and E3 enzymes which target Cyclin D for ubiquitination. It is currently unclear how regulation of the CDC34-SCF complex may modulate Cyclin D proteolysis. In this regard, we have studied the regulation of CDC34 by phosphorylation and by CDC34-associated proteins as well as the interaction of CDC34 with the SCF components, Cul1 and Roc1. Our results suggest that the carboxyl-terminal acidic tail domain of CDC34 does not appear to be critical for its interactions with Cul1 and Roc1 in an in vitro binding assay, while the role of CDC34 phosphorylation on Cul1 and Roc1 binding is still unclear. Currently, our work has focused on identifying the proteins which tightly associate with CDC34 and which are required for DNA replication initiation in Xenopus egg extracts. We are developing strategies to purify the CDC34-associated proteins from human HeLa cell extracts and from Xenopus egg extracts. We predict that these previously unidentified CDC34-associated proteins will play an important role in regulating the ubiquitination of cyclin D in vivo.

14. SUBJECT TERMS

CDC34-SCF, cyclin D, cell cycle, breast cancer

15. NUMBER OF PAGES

45

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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The role of ubiquitin-mediated proteolysis of cyclin D in breast cancer

INTRODUCTION

Cyclin D is a positive regulator of the cell cycle and is found to be highly expressed in breast cancer cells. Cyclin D is post-transcriptionally regulated by the ubiquitin mediated protein degradation pathway. CDC34, a ubiquitin conjugating enzyme, and SCF (Skp1, Cullin, F-box, ring protein), a ubiquitin ligase, are postulated to be the specific E2 and E3 enzymes which target Cyclin D for ubiquitination. It is currently unclear how regulation of the CDC34-SCF complex may modulate Cyclin D proteolysis. Currently we are studying the regulation of CDC34 by phosphorylation, by interactions with the SCF components, Cul1 and Roc1 and by CDC34-associated proteins. I am also developing strategies to identify the CDC34-associated proteins which are required for DNA replication initiation in Xenopus egg extracts. We propose that these previously unidentified CDC34-associated proteins play an important role in regulating the ubiquitination of cyclin D in vivo.

BODY

Task 1

To study the regulation of CDC34 function by phosphorylation in normal and transformed breast cells.

Work done addressing the above specific aim was reported in the previous progress report dated April 30, 2003. It was shown that over expression of wild type CDC34 in 10T-1/2 cells leads to significant increase in the G1 population. The experimental design of these past studies relied on transient transfection assays using CDC34 expressed from mammalian expression plasmids which is limited in its ability to target a large population of cells. We have developed an alternative approach to perform these studies which uses recombinant Adenoviruses expressing human CDC34. We have generated a recombinant Adenovirus which expresses a human tagged wildtype CDC34 which allows for infection of 100% of mammalian cells and high expression of tagged human CDC34 protein. We are currently planning on generating additional recombinant Adenoviruses which express different CDC34 mutant proteins to further address the experiments of this specific aim.

Task 2: Months 1-12

To study the regulation of CDC34-SCF function by compartmentalization during the G1 to S phase transition.

A. Previous studies have shown that CDC34 in a large molecular weight complex is required for initiation of DNA replication in *Xenopus laevis* egg extracts (1). Immunodepletion of CDC34 from Xenopus egg extracts inhibits DNA replication, implying a requirement for CDC34 in the degradation of critical G1 to S phase regulators prior to onset of S phase. Recombinant CDC34 cannot restore DNA replication in CDC34 immunodepleted extracts while a CDC34 protein complex can. This suggested that there were CDC34 associated proteins which played an important role in the initiation of DNA replication. We postulate that the CDC34-associated proteins play an important role in regulating the ubiquitination function of CDC34. These as yet unidentified proteins may regulate the compartmentalization or post-translational modifications of CDC34. These proteins may also be altered in breast cancer cells and may play a role in altering the ubiquitination of CDC34 substrates such as cyclin D during the development of breast cancer.

In order to identify the proteins which tightly associate with CDC34 and are required for DNA replication, we have taken a biochemical approach. We have focused on purifying the CDC34-associated proteins from Xenopus egg extracts which are readily available and which contain robust activity that restores DNA replication to CDC34-immunodepleted egg extracts. A description of my work on this purification follows in the next paragraph. However, a disadvantage of purifying the CDC34-associated proteins from Xenopus extracts is that the Xenopus sequence database is not complete which makes it more difficult to identify proteins based on Mass Spectrometry of biochemically purified proteins. An alternative approach is to use extracts from human cells since the human genome has been fully sequenced. We have shown that extracts from HeLa cells can complement CDC34-immunodepleted egg extracts to restore DNA replication activity. These studies are described in the accompanying manuscript in preparation (2). In summary, these studies demonstrate that fractionated HeLa cell nuclear extracts can restore DNA replication to CDC34-immunodepleted egg extracts and the studies go on to describe a strategy for the purification of the CDC34 complex required for this activity. Once the CDC34-associated proteins are identified, I will determine the role of these proteins on regulating the function of CDC34, in particular, their role on regulating the ubiquitination of cyclin D. I will also determine how these proteins may be altered in breast cancer cells and how this may correlate with cyclin D expression.

I have spent the past several months developing a strategy to purify the CDC34-associated proteins from Xenopus egg extracts. Once this strategy is determined, I will purify these proteins in parallel from both HeLa and Xenopus extracts for identification by either Mass Spectrometry or peptide sequencing. As a first biochemical step, I have studied the use of ammonium sulfate precipitation and found that most of the monomer form of CDC34 (inactive form) can be separated from the functional CDC34 in a high molecular weight complex using a low percentage of ammonium sulfate. The functional CDC34 complex containing all of the replication rescue activity precipitates at a concentration of 20% ammonium sulfate (**Fig. 1** of Appendix).Using this one step, I have

attained a 20-fold purification and a 90% recovery of rescue activity. As a second step, I have utilized ion exchange chromatography. I have used the anion exchanger, DEAE sepharose. I have found that using CDC34-immunodepleted extracts, replication rescue activity elutes exclusively in the 150mM salt elution while the high salt elutions and the unbound fractions do not contain any rescuing activity (**Fig. 2** of Appendix). My results indicate that biochemical fractionation using DEAE yields a 20-fold purification of CDC34-rescuing activity and at least a 30% recovery of activity. My final biochemical purification step is to use immunoaffinity chromatography using a CDC34 affinity purified polyclonal rabbit antibody. I am currently increasing the scale of my purification and combining all three purification steps to isolate enough material for Mass spectrometry analysis.

B. The E2, CDC34, has been shown to function in association with the E3, SCF, to mediate the G1 to S phase transition in budding yeast and mammals. The *CDC34* gene in budding yeast is essential and *cdc34* temperature sensitive (ts) mutant strain arrests in G1 at the non-permissive temperature with a phenotype of multiple buds (3,4). In order to determine whether the phosphorylation of sites within the human CDC34 (hCDC34) acidic tail may be required for functional complementation of the budding yeast ts strain, *cdc34-2*, several human CDC34 clones (**Fig. 3a** of Appendix) were used to perform complementation studies in budding yeast. The results (**Fig. 3b** of Appendix) were reported in the previous progress report. It was reported that the hCDC34 1-200 mutant (deleted of the carboxyl-terminal 36 amino acids), like the active site mutant hCDC34 CL-S, could not complement the ts growth defect of *cdc34-2*. At the time of submission of the previous report, an important control experiment to demonstrate that all the human CDC34 clones were expressed at comparable levels in budding yeast was not yet completed. I have completed this important experiment. Western blot analysis of extracts from transformed budding yeast cells expressing human CDC34 proteins demonstrated that the failure of the hCDC34 1-200 to complement strain *cdc34-2* was not due to a lack of 1-200 protein expression. An immunoblot confirms that all the human CDC34 proteins were expressed at similar levels in budding yeast (**Fig. 3c** of Appendix).

The mammalian SCF/ring complex is composed of F-box binding protein Skp1, the F-box protein Skp2, the scaffold protein CUL1, and the ring finger protein ROC1/Rbx1. The crystal structure of SCF and genetic studies from budding yeast indicate that CDC34 directly associates with the CUL1 and ROC1 proteins (5). Our previous work has also shown that mammalian CDC34 is phosphorylated by Casein Kinase 2 (CK2) (6). Our further studies were focused on trying to understand how CDC34 phosphorylation may regulate its association with CUL1 and ROC1. To study this, I performed in vitro co-immunoprecipitation assays in the presence or absence of CK2 enzyme along with co-translated CUL1-ROC1 proteins, and bacterially-expressed recombinant 6xhis-tagged wildtype CDC34. My preliminary results indicated similar levels of binding of CDC34 and CUL1-ROC1 in the presence and absence of CK2 (**Fig. 4**

of Appendix), suggesting that phosphorylation has no effect on the *in vitro* interactions of CDC34 with CUL1 and ROC1. However, there are caveats to this experiment. It was not possible to determine the percentage of CDC34 that was phosphorylated in the binding assay and we expect that not all the CDC34 was phosphorylated under the conditions utilized. Future studies will determine the exact conditions that are required to ensure that the majority of CDC34 is phosphorylated by CK2 in the binding assay. Additionally, further experiments indicated that in the presence of rabbit reticulocyte lysate (as included in the binding assay to provide the co-in vitro translated CUL1-ROC1 proteins), CDC34 can be phosphorylated even in the absence of any added CK2 enzyme. Therefore, at this time, no conclusions can be drawn from this result and further experiments are required. I also performed further binding studies using WT CDC34, CDC34 1-200 (acidic tail deletion mutant), CDC34 5 PT A (phosphomutant which alters all five CDC34 phosphorylation sites to alanine), and CDC34 5 PT E (mutant which alters all five CDC34 phosphorylation sites to glutamic acid to mimic constitutive phosphorylation) (**Fig. 5** of Appendix). I did not observe a significant difference in the ability of WT and 1-200 CDC34 proteins to bind CUL1 or ROC1 implying that the carboxyl-terminal acidic tail of CDC34 may not play a significant role in the association with CUL1 and ROC1 in an in vitro binding assay. However, I did observe that the CDC34 5 PT A mutant was reduced in its ability to bind to both CUL1 and ROC1 compared to the CDC34 wildtype and the CDC34 5 PT E mutant, suggesting that the phosphorylation of sites within the acidic tail may play some role in the interaction between CDC34 and CUL1-ROC1. Because these in vitro binding results are somewhat contradictory, in the future we will perform *in vivo* binding studies to better examine the role of CDC34 phosphorylation on its association with SCF components.

KEY RESEARCH ACCOMPLISHMENTS

- Development of biochemical purification strategy for CDC34 high molecular weight complex from Xenopus egg and HeLa cell extracts.
- Generation of recombinant Adenovirus expressing wild type human CDC34.

REPORTABLE OUTCOMES

Abstracts:
None

Manuscripts:
None

Awards:
None

CONCLUSIONS

We have generated a recombinant Adenovirus expressing human tagged wildtype CDC34 which will enable us to study the regulation of CDC34 by phosphorylation in normal and breast cancer cells. To further address this aim we need to generate additional recombinant Adenoviruses which would express different CDC34 mutant proteins. Our preliminary results on effect of phosphorylation on human CDC34 interactions with CUL1 and ROC1 are currently inconclusive. We have observed that the Carboxy-terminal acidic tail domain of CDC34 is not required for *in vitro* interactions with CUL1 and ROC1 but phosphorylation of sites within the tail may be required. Further *in vivo* studies will be necessary to clearly understand these interactions. We have developed biochemical purification strategies to purify CDC34 high molecular weight complexes from Xenopus egg and HeLa cell extracts. Now we are focusing on purifying these complexes to identify the CDC34 associated proteins. We postulate that these yet unidentified proteins may be altered in breast cancer cells causing up regulation of cyclin D. We hope that our research will help understand mechanisms of Cyclin D regulation and identify new proteins of therapeutic value.

REFRENCES

1. Yew PR, Kirschner MW. Proteolysis and DNA replication: the CDC34 requirement in the Xenopus egg cell cycle. Science. 1997 Sep 12; 277(5332):1672-6.

2. Block KL, Boyer TG, Bloom J, Pagano M, Yew PR. Biochemical characterization of a human CDC34 complex that rescues DNA replication in CDC34-deficient Xenopus egg extracts. (Manuscript in preparation). *Attached as an appendix*

3. Hereford LM, Hartwell LH. Sequential gene function in the initiation of Saccharomyces cerevisiae DNA synthesis. J Mol Biol. 1974 Apr 15; 84(3):445-61.

4. Schwob E, Bohm T, Mendenhall MD, Nasmyth K. The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in S. cerevisiae. Cell. 1994 Oct 21; 79(2):233-44.

5. Zheng N, Schulman BA, Song L, Miller JJ, Jeffrey PD, Wang P, Chu C, Koepp DM, Elledge SJ, Pagano M, Conaway RC, Conaway JW, Harper JW, Pavletich NP. Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. Nature. 2002 Apr 18; 416(6882):703-9.

6. Block K, Boyer TG, Yew PR. Phosphorylation of the human ubiquitin-conjugating enzyme, CDC34, by casein kinase 2. J Biol Chem. 2001 Nov 2; 276(44):41049-58

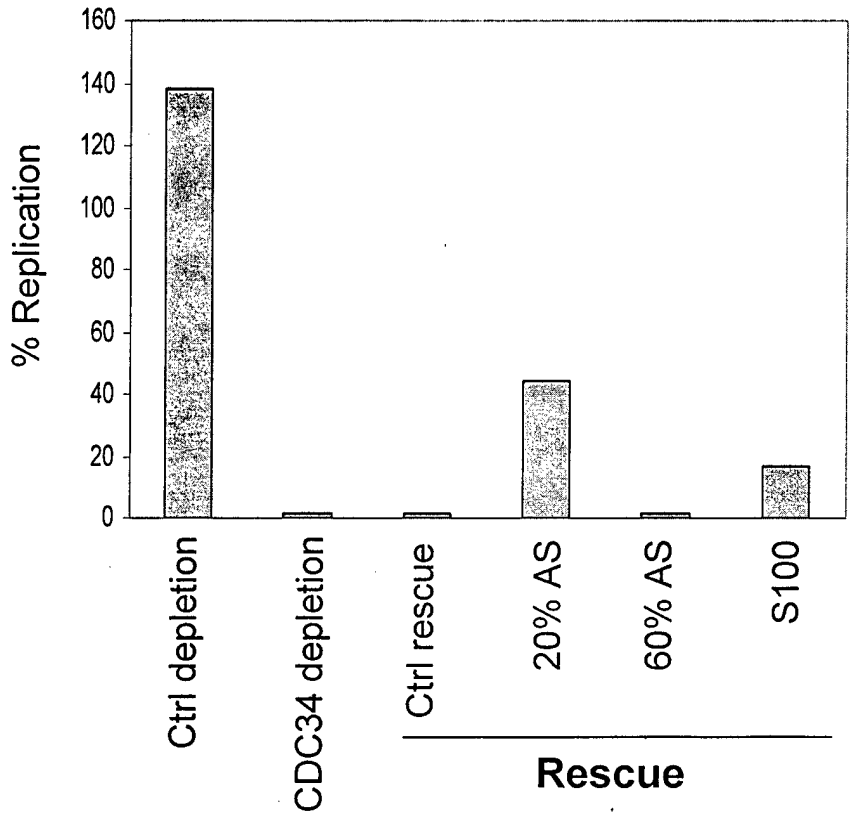

Figure 1. Replication rescue activity is observed in 20% ammonium sulfate precipitate. Depletion-rescue assay where Xenopus egg extracts (LSS) were either control depleted (ctrl depletion) or depleted with anti-CDC34 beads (CDC34 depletion). Xenopus Sperm Chromatin (XSC) was added to the depleted LSS and allowed to replicate with values indicated in % replication of the input template. For rescue, anti-CDC34 beads were incubated with 20 % ammonium sulfate (AS),60 % ammonium Sulfate (AS) and S100 (starting material). The beads were washed mixed with depleted LSS and XSC and allowed to replicate. For control (ctrl) rescue, control beads were incubated with LSS.

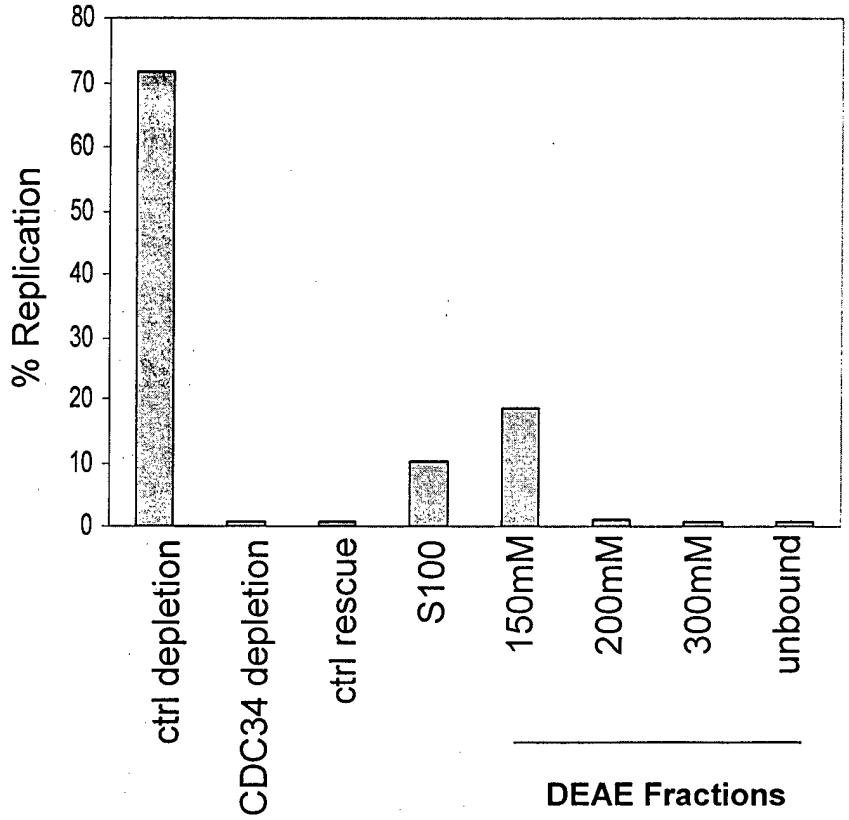

Figure 2. Replication rescue activity is observed in low salt elution of DEAE column purification. S100 (1:5 dilution of Xenopus egg extract, LSS) was applied over a DEAE column at a salt concentration of 50mM potassium chloride (KCl) and the flow through was collected as unbound fraction. The proteins were then eluted using buffers with 150, 200 and 300mM KCl.The collected fractions were then used in a depletion- rescue assay where Xenopus egg extract (LSS) was either control depleted (ctrl depletion) or depleted with anti-CDC34 beads (CDC34 depletion). Xenopus Sperm Chromatin (XSC) was added to the depleted LSS and allowed to replicate with values indicated in % replication. For rescue, anti-CDC34 beads are incubated with S100, 150mM KCl elution, 200mM KCl elution, 300mM KCl elution and the unbound fraction. The beads were washed and to them depleted LSS and XSC were added and allowed to replicate. For the control (ctrl) rescue, control beads were incubated with LSS. Rescue activity was seen in the starting material (S100) and in the 150mM KCl elution. No activity was detected in the high salt elutions or in the unbound fraction.

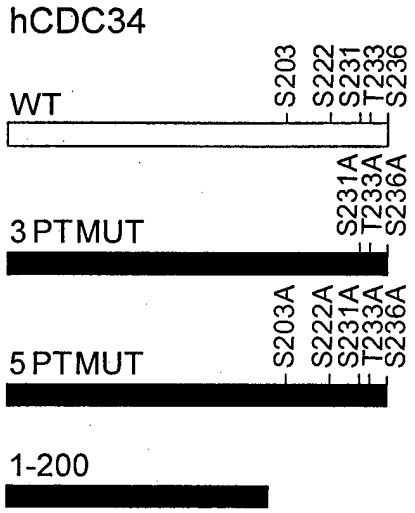

Figure 3a. Schematic of human CDC34 clones used in complementation assay. Human CDC34 (hCDC34) Wild type (WT) is shown with the five phosphorylation sites in the tail domain S203,S222 S231,T233, S236. In a 3 point phosphomutant, three sites, S231,T233,S236, were changed to alanine (3 PT MUT). In another mutant all five sites S203,S222 S231,T233, S236 were changed to alanine (5 PT MUT). The Carboxyl - terminal 36 amino acid residues were deleted in the deletion mutant (1-200).

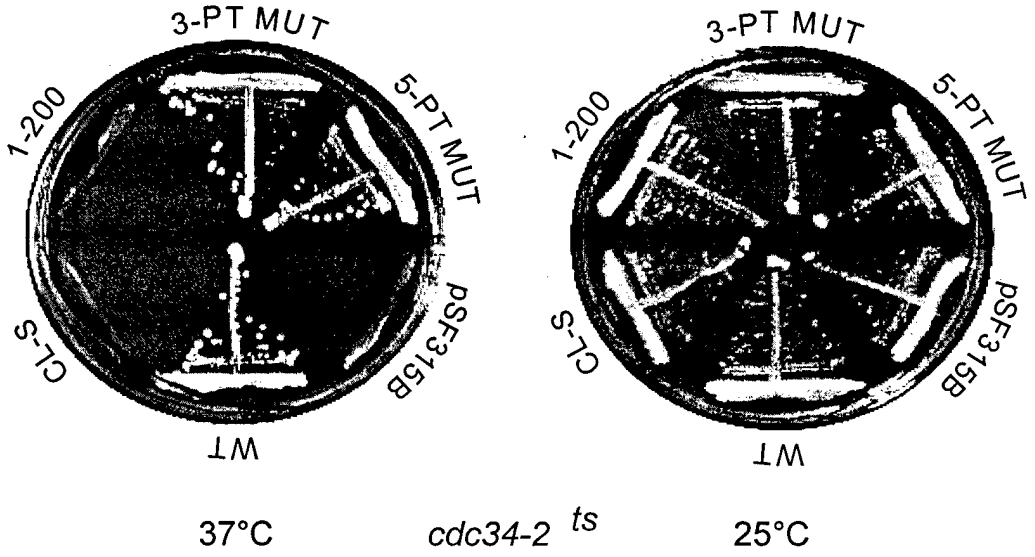

Figure 3b. Amino acids 200-236 of the tail domain of hCDC34 are required for complementation of *cdc34-2* temperature sensitive strain in budding yeast. Human CDC34 (hCDC34) WT and mutants were cloned into plasmid pSF315b for complementation studies using *cdc34-2* temperature sensitive (ts) strain. CL-S is an active site cysteine mutant of hCDC34. pSF315b plasmids were transformed into yeast strains using lithium acetate and then were analyzed for growth at the permissive temperature of 25°C (right) and at restrictive temperature of 37°C (left) on leucine minus minimal plates supplemented with galactose. *Block K et. al.*

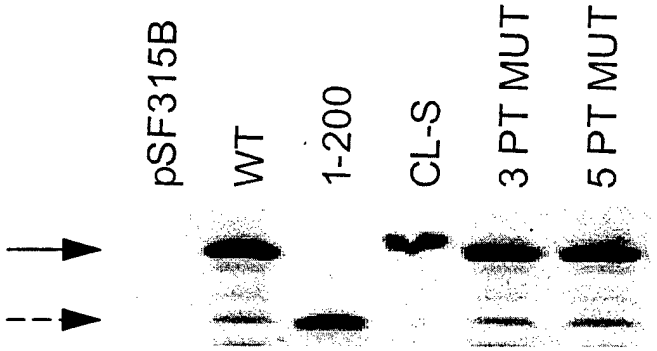

Figure 3c. Human CDC34 proteins are expressed at equivalent levels in the *cdc34-2* temperature sensitive strain of budding yeast. pSF315b plasmids were transformed into yeast strains using lithium acetate. The transformed cells were induced to express proteins and lysates were prepared. Equal amounts of proteins were loaded in each lane. Full length CDC34 protein bands are indicated by the arrow and the dotted arrow indicates the truncated CDC34 protein. Human CDC34 is not observed in cells transformed with pSF315b vector alone.CDC34 could be detected in cells transformed with plasmids expressing human Wild Type CDC34(WT), deletion mutant (1-200), active site mutant (CL-S), 3 point phosphomutant (3 PT MUT) and 5 point phosphomutant (5 PT MUT).

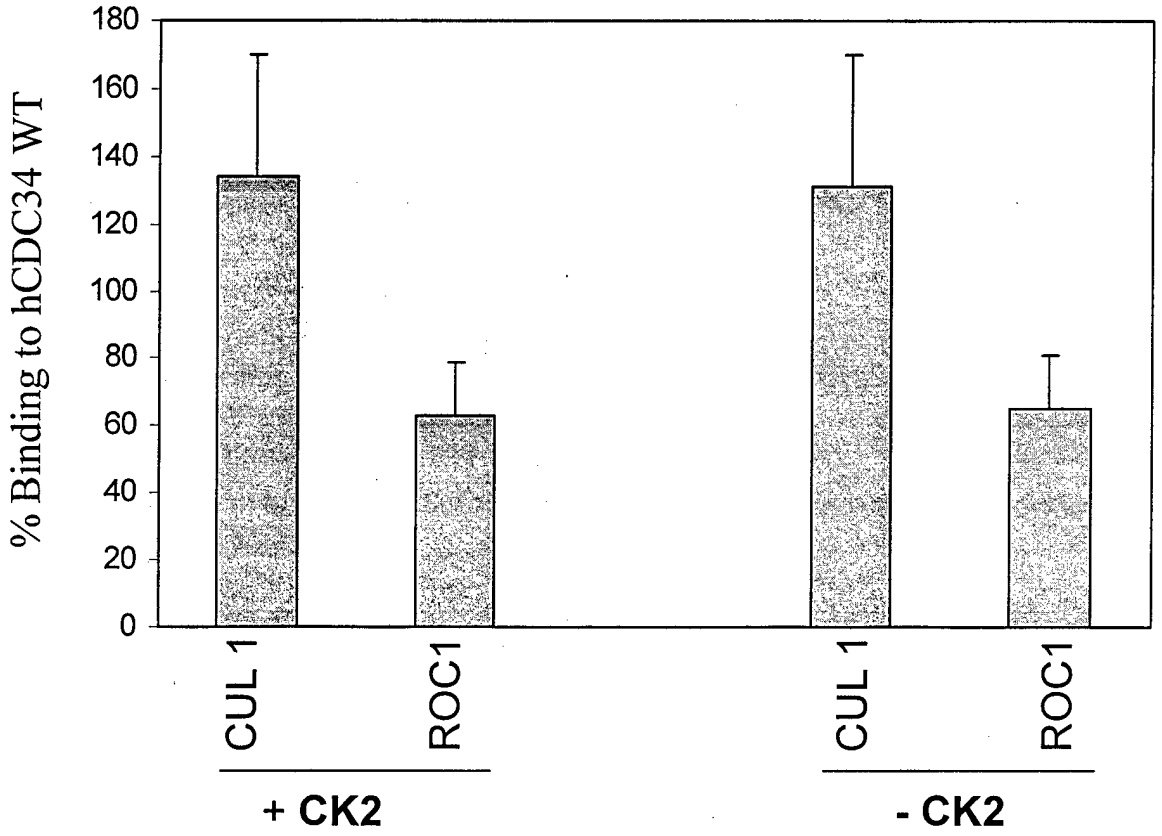

Figure 4. Phosphorylation of CDC34 by CK2 does not apper to influence the interaction of CDC34 with CUL1 and ROC1. *In vitro* Kinase and Co-Immunoprecipitation assay. Purified 6xhis humanCDC34 wild type (hCDC34 WT) was incubated first with Casein Kinase 2 (CK2) (left) or with control buffer (right) and then with co-in vitro translated ^{35}S methionine labeled CUL1-ROC1. The samples were then immunoprecipitated with CDC34 antibody. Samples were analyzed by SDS-PAGE and phosphoimager analysis. Results are shown as percentage CDC34 bound CUL1 or ROC1.

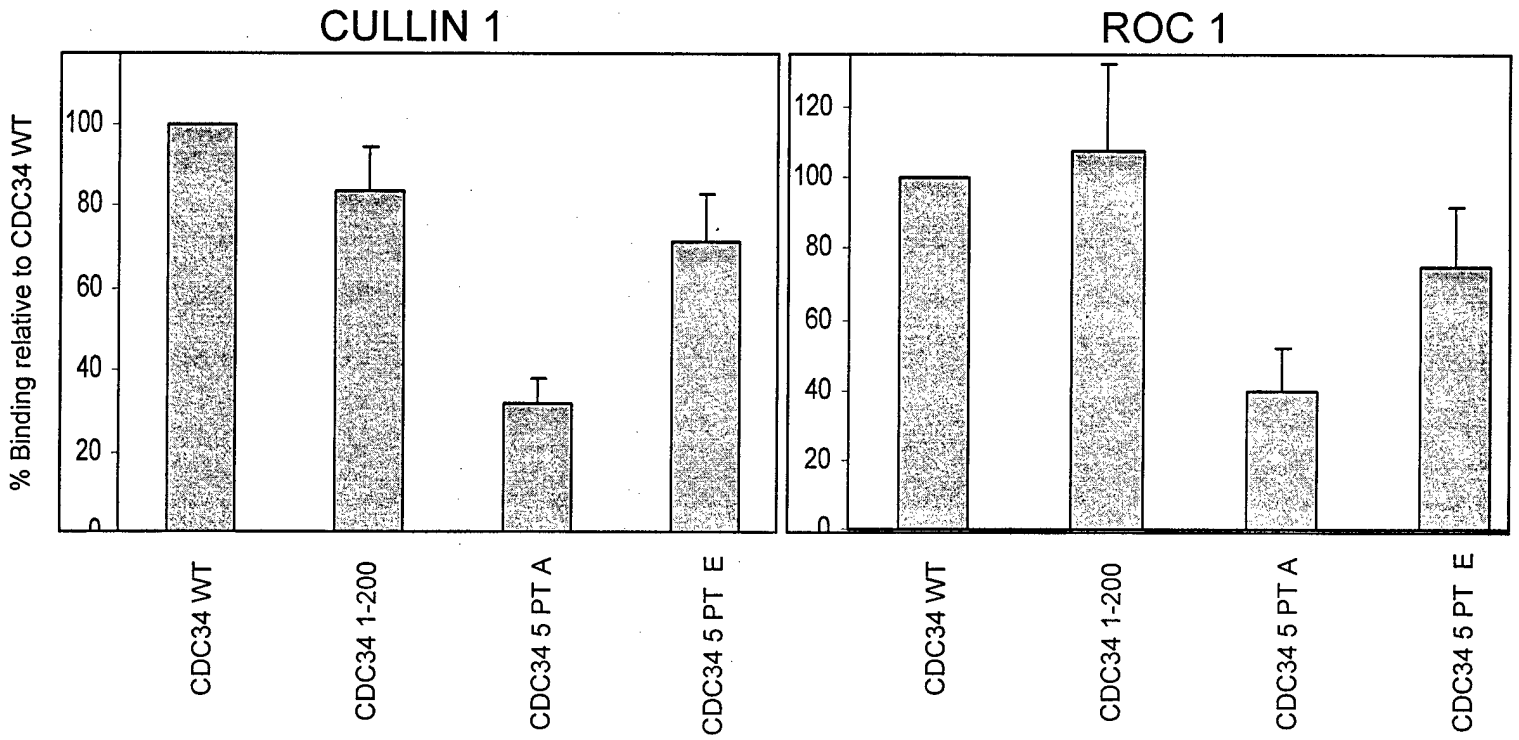

Figure 5. The acidic tail domain of hCDC34 is not required for binding to CUL1 and ROC1 but the phosphorylation of sites in the tail domain may be required. Purified 6xhis CDC34 wild type (CDC343 WT), deletion mutant(CDC34 1-200), phosphomutant (CDC345 PT A) and glutamic acid mutant (CDC345 PT E) were incubated with co-in vitro translated ^{35}S methionine labeled CUL1-ROC1. The samples were then immunoprecipitated with CDC34 antibody. Samples were analyzed by SDS-PAGE and phosphoimager. Results are shown as the percentage binding of CDC34 mutants to CUL1-ROC1 calculated relative to WT.

BIOCHEMICAL CHARACTERIZATION OF A HUMAN CDC34 COMPLEX THAT RESCUES DNA REPLICATION IN CDC34-DEFICIENT *XENOPUS* EGG EXTRACTS

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Running Title: Characterization of Human Cdc34 DNA Replication Activity

SUMMARY

Previous studies have shown that Cdc34 in a large molecular size complex is required for the initiation of DNA replication in eggs from *Xenopus laevis*. Our studies characterize a human Cdc34 complex from HeLa cells which is able to complement DNA replication in Cdc34-immunodepleted *Xenopus* egg extracts. We find that while immunoaffinity purified HeLa nuclear extract cannot restore DNA replication to Cdc34-depleted *Xenopus* extract, immunoaffinity purified HeLa nuclear extract passaged over phosphocellulose can restore DNA replication to Cdc34-depleted extracts. We further fractionated HeLa nuclear extract passaged over phosphocellulose using DEAE anion exchange, gel filtration, and immunoaffinity chromatography and found that only Cdc34 present in a large molecular size complex of ~400 kDa was able to rescue DNA replication in Cdc34-depleted *Xenopus* extract. Immunoaffinity purification of the Cdc34 complex from partially purified HeLa nuclear extract indicated that SCF components, Cul1 and $p19^{Skp1}$, were not tightly associated with human Cdc34, despite the ability of this Cdc34 complex to restore DNA replication in Cdc34-depleted *Xenopus* extracts. Further, the addition of recombinant human Cdc34 and $SCF^{p45Skp2}$ was not sufficient to restore DNA replication to Cdc34-depleted *Xenopus* extract. These studies suggest that human Cdc34 in association with as yet unidentified Cdc34-interacting proteins can complement DNA replication in Cdc34-deficient *Xenopus* egg extract.

INTRODUCTION

Cell cycle progression and homeostasis require ubiquitin–dependent proteolytic degradation. Regulated protein degradation involves a pathway in which ubiquitin is activated, in an ATP dependent manner, for transfer to a substrate. Subsequently the poly-ubiquitinated substrate is recognized by the 26S proteosome and destroyed (Jentsch, 1992,Ciechanover, 1994). These cascades of events begin when ubiquitin-activating enzyme (E1) forms a thioester bond to the carboxy-terminus of ubiquitin. Ubiquitin is transferred, through thioesterification, to a member of a family of ubiquitin-conjugating enzymes (E2). Finally, the activated ubiquitin will be transferred to a lysine residue on the substrate directly by the E2 or it will be passed to the ubiquitin ligating enzyme (E3) and then to the substrate. E3s bind the substrate directly, suggesting they provide specificity in ubiquitin–dependent proteolytic degradation. A requirement for ubiquitin–dependent protein degradation at the G1/S transition was first observed in *S. cerevisae* where a temperature sensitive (ts) mutant arrested at the initiation of DNA replication because p40Sic1, an inhibitor of S phase promoting Clb/Cdc28 complexes, was unable to be degraded (Schwob, 1994).

In Xenopus egg extracts, depletion of a ubiquitin conjugating enzyme, Cdc34, abrogated DNA replication initiation suggesting a role for Cdc34 at the onset of DNA replication, presumably through its function in protein degradation (Yew and Kirschner 1997). Purified

[3] The abbreviations used are: AP Cdc34, affinity purified Cdc34; BSA, bovine serum albumin; CDC, cell division cycle; Ctrl, control; CK2, Casein Kinase 2; CK1, CSA, chicken serum albumin; DTT, dithiothreitol E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzymes; E3, ubiquitin ligating enzyme; hCK2β, human Casein Kinase 2β; hCdc34, human Cdc34; Ig, immunoglobulin; IP, immunoprecipitation; SCF, Skp1, Cullin, F-box; ScCdc34p, Cdc34p in *S. cerevisiae*; MUT, mutant; NEXT, nuclear extract; No Depl, not depleted; NRS, normal rabbit serum; PCFT, phosphocellulose flow through; RIgG, rabbit immunoglobulin; LSS, low speed supernatant; PCR, Polymerase Chain Reaction; RER, energy-regenerating system; RU, rescuing units; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; UBC, ubiquitin conjugating enzyme; WCE, whole cell extract; WT, wildtype.

Cdc34 alone is not sufficient to rescue DNA replication activity in the Cdc34 immunodepleted egg extracts, suggesting that Cdc34 in association with other proteins are required. In mammals, Cdc34 interacts and functions with different ubiquitin ligating enzymes targeting substrates that play a role in cell cycle progression, signal transduction, and development (Tyers M 2000, Koepp 1999). The most characterized ubiquitin ligating enzyme that functions with Cdc34 is p19Skp1/Cul1/p45Skp2 (F-box)/Roc1 (ring) or SCF/ring complex.

We have also identified the regulatory (β) subunit of human Casein Kinase 2 (CK2) as a novel Cdc34 interacting protein through a yeast two hybrid screen. We have shown that Cdc34 is a phosphoprotein that immunoprecipitates with kinase activity. This associated kinase activity is biochemically characteristic of CK2 (Block et al., 2001). CK2 activity has also been implemented in cell cycle progression, signal transduction, and more recently, protein stability (Uhle et al., 2003, Semplici, oncogene 2002, Allende and Allende 1998, Guerra and Issinger 1999, Pinna and Meggio, 1997). Although protein degradation has been linked to cell cycle progression for some time now, ongoing investigations are identifying novel interacting proteins and post-translational modifications of the ubiquitin conjugating enzymes as well as the ubiquitin ligating enzymes which are continuing to provide insights as to the levels of complexity of protein degradation and their regulation in higher eukaryotes. The Xenopus system has proven to provide mechanistic insights to cell cycle events. It is currently unclear what degradation event/events are required for DNA replication in a vertebrate. Here we describe the partial purification and biochemical characterization of the Cdc34-associated protein complex in HeLa extracts required for DNA replication activity in Cdc34-immunodepleted *Xenopus* egg extracts.

EXPERIMENTAL PROCEDURES

Sucrose gradient centrifugation. Isokinetic 10-40% sucrose gradients (HKME buffer: 50mM Hepes pH 7.2, 100mM KCL, 5mM $MgCl_2$, 0.2 mM EDTA, 2mM DTT, 1x protease inhibitors) were poured in a two chamber gradient connected to a peristaltic pump. Gradients were poured by displacement method with a glass capillary tube at the bottom of the centrifugation tube. The glass tube was carefully removed vertically afterwards without disturbing the gradient. A mixture of standards including Thyroglobulin, Ferritin, Catalase, Aldolase, BSA and Lysozyme, each 375 ug, were layered onto the gradient. HeLa extracts were dialyzed in buffer D- (20mM Hepes pH 7.9, 100mM KCl, 5mM B-mercaptoethanol, 0.5 mM PMSF, 0.1mM EDTA). 5 mgs of HeLa whole cell extract (HeLa WCE), 5 mgs of HeLa nuclear extract (HeLa NEXT), or 4.2 mgs of HeLa phosphocellulose flow through (HeLa PCFT) were layered onto the gradient. Samples were subjected to centrifugation at 37,500 rpm for 17.5 hours. Fractions were collected by cutting 0.5 mL aliquots from the top of the gradient. 52.5 uL of each fraction was added to sample buffer and resolved on a SDS-Page gel and immunoblots were performed. In parallel, 0.5 mL fractions of standard markers were resolved on SDS-PAGE and subsequently coomassie blue stained and calibration curves plotted.

Immunodepletion/Rescue studies. Xenopus laevis interphase low speed supernatant (LSS) extracts were generated as previously described (Yew Kirscner1997). Demembranated sperm nuclei were prepared as described (J.J. Blow and R.A. Laskey, Cell 47 577 1986). DNA replication was measured by tricloroacetic acid precipitation of radiolabeled fragments (Yew and Kirchner 1997) and presented as percent replication of the input template (5ng/ul). Antibodies were covalently cross-linked to protein A (pA)-sepharose(Seph) or pA-affiprep as described

(Yew and Kirschner, 1997). Cyclohexamide (0.1mg/ml) and energy-regenerating system (RER) was added to LSS before replication was assayed. Immunodepletetion was performed by mixing 1 volume of LSS and 1.25 volume antibody coupled pA-seph beads for 2-3 hours at 0^0C with resuspension every 10 minutes. Rescue assays were performed by incubating 8uL coupled pA-affiprep beads with indicated *X. laevis* LSS or indicated fractionated mammalian extracts overnight at 4^0C in the presence of 1mM ATP. The beads were washed 3x with XB-(10mM Hepes pH 7.2, 100mM KCL, 0.1mM $CaCl_2$, 1mM $MgCl_2$). Immunodepleted LSS was added to washed rescue beads and assayed for replication as described. For SCF/ring, CK2 rescue studies, 200nM of bacterial 6xhis Cdc34 with 200nM of each baclovirus SCF/ring component indicated or purified enzyme CK2 was added to immunodepleted LSS and assayed for replication. Rescuing units are calculated as %replication of rescue sample/% replication of control rescue (Ctrl).

Immunoprecipitation-western (IP-western) and Direct Westerns. 2mgs of mammalian HeLa Phosphocellulose flow through (HeLa PCFT) was incubated overnight with antibody coupled Cdc34 pA-affiprep beads or control serum (NRS) coupled pA-affiprep beads. Beads were washed 3x with XB- and boiled in Laemmli sample buffer and resolved by SDS-Page. Immunoblots were performed on the immunoprecipitated material or on 50 ug of indicated cell lysate per lane. Immunoblots were incubated with affinity purified Cdc34 anti-body, Cullin 1 antibody (neomarkers) or p19Skp1 (Santa Cruz). The immunoblots were washed and incubated with pA-coupled horse radish peroxidase (Biorad) followed by chemiluminescence using ECL reagent (Amersham-Parmacia). Other immunoblots were incubated with Casein Kinase 2 (calbiochem) or p45Skp2 (Santa-Cruz). Immunoprecipitation from HeLa PCFT/DEAE 150mM

KCl fraction was performed by taking 10mgs of the purified 150mMKCl DEAE bump/IP and incubating the fraction with affinity purified Cdc34 antibody covelantly coupled to pA-affiprep or RigG covelantly coupled to pA-affiprep. The immunoprecipitate was washed (XB-/250mMKCl) and eluted by acid base. The eluent was concentrated and 1/5th was resolved on SDS-PAGE for western blot analysis and the remaining 80% was resolved on SDS-PAGE and subjected to silver stain.

Gel Filtration Fractionation/silver stain: DEAE 250mMKCL/SDEX200 purification: HeLa PCFT was dialyzed in DEAE binding buffer (50mM Tris pH 8.0, 50 mM KCL, 5mM $MgCl_2$, 0.2 mM EDTA, 10% Glycerol, 1mM DTT). 133.6 mgs of HeLa PCFT in binding buffer was loaded onto DEAE and washed in binding buffer. The flow through was collected and subsequent washes of eluting buffer (50mM Tris pH 7.7, 250 mM KCl or 600 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 10% Glycerol and 1mM DTT were passed over the column and collected. Pooled peaks were combined and each fraction was assayed for protein by western analysis. In addition, each fraction was assayed for DNA replication/rescue (data not shown) activity. Subsequently, the 250 mM KCl fraction was equilibrated in 150 mM KCL, concentrated and 22 mgs was passed over a HiLoad 16/60 SDex 200 column (Pharmacia). Fractions were collected and concentrated. 1/60th of each fraction or 50ug of indicated cell lysate was used for immunoblot analysis. 1/4th of each fraction was used in a rescuing assay as described. 3uL of each purified fraction was analyzed by silver staining. **DEAE 150mM purification:** HeLa PCFT was dialyzed in DEAE binding buffer (50mM Tris pH 8.0, 50 mM KCL, 5mM $MgCl_2$, 0.2 mM EDTA, 1mM DTT) and 158mgs HeLa PCFT loaded onto the DEAE column. The column was washed with binding buffer, flow through fraction (FT) and subsequently eluted with 50mM Tris

pH 7.2, 150 mM KCl, 5mM $MgCl_2$, 0.2 mM EDTA or 50mM Tris pH 7.2, 300 mM KCl, 5mM $MgCl_2$, 0.2 mM EDTA or 50mM Tris pH 7.2, 600 mM KCl, 5mM $MgCl_2$, 0.2 mM EDTA. The peak of each fraction was pooled, dialyzed in HKME (50mM Hepes pH 7.2, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA), and concentrated. 25uL of each fraction was used in a western analysis and $1/41^{th}$ of each fraction was used in a rescue assay. 3uL of each purified fraction was analyzed by silver staining.

RESULTS

Immunoaffinity purified HeLa nuclear extract passaged over phosphocellulose can restore DNA replication in Cdc34-immunodepleted *Xenopus* egg extracts. It has been characterized in *Xenopus laevis* that a low speed supernatant (LSS) egg extract immunodepleted of Cdc34 cannot support DNA replication onset. Incubating LSS with pA-affiprep covelantly coupled anti-Cdc34 beads rescues the Cdc34 depleted egg extract but not purified bacterial Cdc34 alone (Yew and Kirschner 1997). To determine the ability of a mammalian cell extract to rescue Cdc34 immunodepleted Xenopus egg extract, we incubated 1100 ug of HeLa nuclear extract (HeLa NEXT) or HeLa nuclear extract passaged over a phosphocellulose column (HeLa PCFT) with pA-affiprep covelantly coupled anti-Cdc34 beads. The results show that LSS incubated with normal rabbit serum covelantly coupled to pA-sepharose beads (Ctrl Depl) support DNA replication (Fig 1 left panel). However, depletion of Cdc34 with antibody covelantly coupled to pA-sepharose incubated in LSS (Cdc34 Depl) does not support DNA replication (Fig 1 left panel). This Cdc34 immunodepleted extract was incubated with pA-affiprep beads covelantly coupled to Cdc34 or normal rabbit serum that had been incubated in LSS, HeLa NEXT, or HeLa PCFT. Normal rabbit serum incubated with LSS could not rescue DNA replication in the Cdc34

depleted egg extract although anti-Cdc34 beads incubated in the LSS could (Fig 1 right panel, Ctrl and XlLSS respectively). pA-affiprep coupled anti-Cdc34 beads incubated in a HeLa PCFT can rescue DNA replication in a Cdc34 depleted egg extract whereas anti-Cdc34 beads incubated in a nuclear extract could not (HeLa NEXT) (Fig 1 right panel). These results suggest that mammalian HeLa PCFT extract incubated with pA-affiprep beads covelantly coupled to anti-Cdc34 can rescue DNA replication in a Cdc34 depleted Xenopus egg extract. Importantly these findings also suggest that Cdc34 in a HeLa nuclear extract may be associated with an inhibitor of DNA replication onset in Xenopus LSS egg extract and that passage of a HeLa nuclear lysate over a phosphocellulose column restores DNA replication rescuing activity.

The Cdc34 associated DNA replication activity in HeLa extracts is found in a higher molecular weight complex. We wanted to purify and characterize the Cdc34 associated DNA replication rescuing activity in mammalian cells. We further fractionated the HeLa Phosphocellulose Flow Through (PCFT) by passing it onto a DEAE anion exchange column and took three fractions, the flow through, a 250mM KCl bump and a 450 mM KCl bump. We examined each fraction by western analysis for Cdc34p and known Cdc34 interacting proteins (Fig 2A). SCF components, Cullin1, p45Skp2, and p19Skp1 all co-fractionated with Cdc34 in the 250mM KCl bump. However, another known Cdc34 interacting protein Casein Kinase 2 (CK2) did not (Fig 2A). Each fraction was tested for Cdc34 rescuing activity as described in Fig 1. The HeLa PCFT/DEAE 250 mM KCl fraction showed DNA replication rescuing activity in the Xenopus Cdc34 depleted egg extract (data not shown). We further fractionated the 250 mM KCl bump over a SDEX 200 sizing column. Each fraction of the SDEX 200 column was collected, concentrated resolved on a SDS-Page gel and transferred to nitrocellulose and

analyzed by western analysis for Cdc34p and other known Cdc34 interacting proteins (Fig 2B). The molecular weight sizes listed above represent the elution of standards used to calibrate the column (Fig 2B above top panel). These western results suggest that endogenous Cullin 1 in fractionated HeLa extracts is only found in a higher molecular weight complexes of 232-440 kDa. Cullin1 encodes for a protein of ~85kDa. p45Skp2 is also found in several fractions of higher molecular weight from ~67-660 kDa. p19Skp1 is spread over many fractions from 20-660 kDa. (Fig 2B). This is consistent with other studies that show that fractionation of p19Skp1 and p45Skp2 in HeLa cells over a gel filtration column exist in many different fractions (Raymond et al., 1998). Endogenous hCdc34 in fractionated HeLa cells is found predominately in a free form, however 1-3 percent is found in higher molecular weight complexes of 232-660kDa. This is consistent with fractionated S100 extracts from Xenopus laevis following Cdc34 protein (Yew and Kirschner, 1997). There is a modified form of Cdc34 that migrates slower in SDS-Page in fractions 9 and 10. We do not think this modified Cdc34 species is a Cdc34 phosphorylation form as we do not observe a shift of Cdc34 protein in our phosphorylation studies (Block et al., 2001). This shift may represent a ubiquitinated species of Cdc34 as Cdc34 is autoubiquitintated in vitro and in vivo (Banerjee et al., 1993, Goebl et al., 1995, Ohta et al., 1999, Seol et al., 1999, Chen et al., 2000). We analyzed each fraction by silver staining to see the complexity of the purified SDEX fractions (Fig 2C). In addition, each fraction was tested for Cdc34 rescuing activity in Xenopus Cdc34 depleted egg extracts as previously described. The results show that LSS not depleted and LSS incubated with normal rabbit serum covelantly coupled to pA-sepharose beads support DNA replication (Fig 2D left panel). However, depletion of Cdc34 with antibody covelantly coupled to pA-sepharose incubated in LSS does not (Fig 2D left panel). This Cdc34 immunodepleted extract was incubated with pA-affiprep beads

covelantly coupled to Cdc34 or normal rabbit serum that had been incubated in LSS or each eluted SDEX 200 fraction. Normal rabbit serum covelantly coupled pA-affiprep beads incubated with LSS could not rescue DNA replication in the Cdc34 depleted egg extract although anti-Cdc34 coupled pA-affiprep beads incubated in the LSS could (Fig 2D right panel). pA-affiprep coupled anti-Cdc34 beads incubated in HeLa PCFT/DEAE 250mM KCl/SDEX200 fractions indicate that the Cdc34 associated rescuing activity is found in fractions 10,11 of the SDEX200 column (Fig 2D right panel noted by an astrick (*)). Importantly, these results suggest that the Cdc34 associated rescuing activity is found with the smaller percent of Cdc34 in a higher molecular weight complex of ~ 400 kDa rather than the higher percent of Cdc34 that is in a monomer form.

Fractionation of HeLa WCE, HeLa NEXT, HeLa PCFT extracts by sedimentation. To characterize endogenous Cdc34 in non fractionated HeLa cell extracts, we prepared a HeLa whole cell extract (HeLa WCE) and resolved it on a sucrose gradient. A linear sucrose gradient was prepared so that the 40% was on the bottom of the gradient and 10% on the top. Standards were run in parallel and 0.5 mL fractions were cut from the top of the gradient and subjected to a SDS-Page gel and subsequently coomassie blue stained. The molecular weight sizes listed above (Fig 3A.) represent the fractionation of standards used to calibrate the sucrose gradient. Western analysis was performed on each fraction of the HeLa WCE using antibodies against Cdc34 (Fig 3A middle panel) and known Cdc34 interacting proteins, Casein Kinase 2 (CK2) and p19Skp1 (Fig 3A top panel and bottom panel respectively). These results suggest that the majority of Cdc34 (~90%) is free and only a small percent of Cdc34 is present in a large molecular size complex of 275-300 kDa. CK2 and p19Skp1 proteins can also be detected in the Cdc34 higher

molecular weight fraction. Because the results in Fig 1 suggest that Cdc34 in a HeLa nuclear extract (HeLa NEXT) may be associated with an inhibitor of DNA replication activity, we passed a HeLa PCFT and a HeLa NEXT over a linear sucrose gradient as just described to determine if there was a shift in the Cdc34 protein or Cdc34 high molecular weight protein complex. Western analysis on each of these extracts did not show a difference in the free Cdc34 (Fig 3B). However, we observed that Cdc34 in the higher molecular weight fractions in a HeLa NEXT appeared to shift to fractions of lower molecular weight when passaged over a phosphocellulose column. These results suggest, a protein that associates with Cdc34 in the HeLa nuclear extract is fractionated away or dissociated when passed over a phosphocellulose column. This, however, needs to be further investigated. We cannot rule out that the higher molecular weight complex of Cdc34 breaks apart when further fractionated over the phosphocellulose column. This fractionated HeLa PCFT extract does have Cdc34 rescuing activity in a Cdc34 depleted Xenopus egg extract as shown in Fig 1. Cdc34 fractionated in HeLa PCFT by gel filtration indicated Cdc34 in higher molecular weight complexes of 232-660kDa. The difference of molecular weight of Cdc34 in a HeLa WCE by sedimentation versus Cdc34 HeLa PCFT over gel filtration suggest that the Cdc34 protein complex is not spherical in nature rather it is globular.

HeLa PCFT passed over a DEAE anion exchange column and eluting at 150mM KCl separates the small percent of Cdc34 associated DNA replication activity from the majority of Cdc34 free form. We passed HeLa PCFT over a DEAE anion exchange column and eluted with more KCl bumps to see if we could further fractionate the Cdc34 associated rescuing activity. We examined, by western analysis, the flow through, a 150mM KCl bump, a 300 mM

KCl bump and a 600 mM KCl bump for Cdc34 and other Cdc34 interacting proteins. We found that a very small percent of Cdc34 eluted and could be detected in the 150 mM KCl fraction, whereas the majority of other known Cdc34 interacting proteins, Cul1/Skp2 and Skp1 did elute in the 150 mM KCl bump (Fig 4A). The majority of Cdc34 eluted at the 300 mM KCl bump (Fig 4A). We ran each fraction on a SDS-Page gel and analyzed the complexity of these fractions by silver stain (Fig 4B). We then tested each fraction for Cdc34 associated DNA replication activity. The results show that LSS not depleted and LSS incubated with normal rabbit serum covelantly coupled to pA-sepharose beads support DNA replication. However, depletion of Cdc34 with antibody covelantly coupled to pA-sepharose incubated in LSS does not (Fig 4C left panel). This Cdc34 immunodepleted extract was incubated with pA-affiprep beads covelantly coupled to Cdc34 or normal rabbit serum that had been incubated in LSS, or each eluted DEAE fractions. Normal rabbit serum coupled pA-affiprep beads incubated with LSS could not rescue DNA replication in the Cdc34 depleted egg extract although anti-Cdc34 coupled pA-affiprep beads incubated in the LSS could (Fig 4C right panel). pA-affiprep coupled anti-Cdc34 beads incubated in the HeLa PCFT/DEAE fractions indicate that the Cdc34 associated rescuing activity is found in the 150 mM KCl fraction (Fig 4C right panel noted by an astrck (*)). This purification step is very efficient, as it appears to separate the small percent of Cdc34 that is associated with DNA replication rescue activity from the bulk of Cdc34 that is not complexed and does not exhibit any associated DNA replication activity.

Known Cdc34 associated proteins are not sufficient for rescuing DNA replication in a Cdc34 depleted Xenopus egg extract. A requirement for proteolysis and DNA replication has well been established. It has been shown through this study and in studies using Xenopus egg

extracts that Cdc34 in a higher molecular weight complex and not free Cdc34 exhibits associated DNA replication activity. It is also known that Cdc34 associates and functions with its E3, the SCF/ring complex in regulated protein degradation via the ubiquitin pathway. In addition, components of the SCF complex, Cullin1, p45Skp2 and p19Skp1 co-fractionates with Cdc34 associated rescuing activity in the DEAE 150mM KCl fraction. It was therefore important to determine if the SCF complex is necessary and or sufficient for rescuing a Cdc34 depleted Xenopus egg extract. We took two approaches to address this question. First, we know that pA-affiprep coupled to Cdc34 antibodies and incubated in a HeLa PCFT immunoprecipitates proteins that associate with Cdc34 that are both necessary and sufficient for DNA replication rescuing activity in a Cdc34 depleted Xenopus egg extract. Therefore, we immunoprecipitated 2 mgs of HeLa PCFT with pA-affiprep coupled to Cdc34 antibodies or pA-affiprep coupled to normal rabbit serum and examined the immunoprecipitate by western analysis (IP-western). The results show that endogenous CUL 1 and Skp1 are not detectable by western analysis in the co-precipitate using anti-Cdc34 coupled pA-affiprep beads although Cdc34 is easily detected (Fig 5A lanes 3,4). HeLa PCFT or HeLa PCFT/DEAE 250 mM KCl lysate were loaded as western controls (Fig 5A lanes 1,2 respectively). Importantly, we next took the HeLa PCFT/DEAE 150 mM KCl fraction that exhibited high Cdc34 rescuing activity and immunoprecipitated (IP) Cdc34. This fraction was enriched for Cul1 and p19Skp1 and only the small percent of Cdc34 in a complex required for DNA replication rescuing activity. We immunoaffinity purified Cdc34 antibodies (AP-Cdc34) and covelantly coupled AP-Cdc34 or RIgG to pA-affiprep beads as described in the methods. We incubated the HeLa PCFT/DEAE 150mM KCl fraction to AP-Cdc34 or RIgG coupled beads, washed the beads and eluted the Cdc34 immunoprecipitate. The eluted immunoprecipitate was concentrated and and $1/5^{th}$ was resolved by SDS-Page analyzed by

western analysis. The RIgG precipitate did not immunoprecipitate Cul1/Cdc34 nor p19Skp1. The Cdc34 immunoprecipitate easily detected Cdc34 however, Cul1 and p19Skp1 were not detected by western analysis (Fig 5B). This suggests that known SCF proteins that function with Cdc34 do not immunoprecipitate with Cdc34 nor with Cdc34 rescuing activity. Finally, we tested the ability of purified bacterially expressed 6xhisCdc34 with purified baclovirus SCF/Roc1 or another known Cdc34 interacting protein, CK2, to rescue a Cdc34 depleted Xenopus egg extract. The results show that LSS not depleted and LSS incubated with normal rabbit serum covelantly coupled to pA-sepharose beads support DNA replication (Fig 5C left panel). However, depletion of Cdc34 with antibody covelantly coupled to pA-sepharose incubated in LSS does not (Fig 5C left panel). This Cdc34 immunodepleted extract was incubated with pA-affiprep beads covelantly coupled to Cdc34 or normal rabbit serum that had been incubated in LSS. Normal rabbit serum pA-affiprep coupled beads incubated with LSS could not rescue DNA replication in the Cdc34 depleted egg extract although anti-Cdc34 pA-affiprep coupled beads incubated in the LSS could (Fig 5C right panel). Further, 200nM of 6xHisCdc34 with 200nM baclovirus purified Cul1/ROC1 (Fig 5C lane 6) or 200nM of 6xHis Cdc34 with 200nM baclovirus purified Cul1/ROC1/Skp1/Skp2 (Fig 5C lane 7) or 6xHis Cdc34 with 200nM purified enzyme of Casein Kinase 2 (CK2) (Fig 5C lane 8) was added to the Cdc34 depleted egg extract. We did not detect any rescuing activity over background in these studies. This suggests that the SCF/ring complex alone is not tightly associated with Cdc34 and that these known interacting proteins are not sufficient when combined with Cdc34 to rescue a Cdc34 depleted egg extract although anti-Cdc34 coupled pA-affiprep beads incubated in a HeLa PCFT is. Taken together, this suggests that there are yet unidentified associated proteins of Cdc34 that are required for the onset of DNA replication in Xenopus.

Potential Cdc34 interacting proteins. We have fractionated Cdc34 rescuing activity and our fractionation protocol is shown schematically in Figure 6A. We have found that HeLa PCFT over a DEAE column and eluting at 150mM KCl followed by immunoaffinity with Cdc34 antibody exhibits the highest DNA replication Cdc34 rescuing activity as assayed in a Cdc34 Xenopus Cdc34 depleted egg extracts. Because it appears that known Cdc34 interacting proteins are not sufficient for our Cdc34 rescuing activity, we wanted to next examine the HeLa PCFT/DEAE 150mM fraction for candidate Cdc34 interacting proteins. We took the concentrated and eluted fraction from the Cdc34 immunoprecipitation PCFT/DEAE 150mM fraction and resolved it on a SDS-PAGE gel and performed silver stain. The results indicate candidate Cdc34 interacting proteins that are not found in the RIgG immunoprecipitation (Figure 6B). We have identified seven candidate Cdc34 interacting proteins with approximate molecular weights of; 56 kDa, 60 kDa, 90 kDa, 110 kDa, 120 kDa, 150 kDa, and 220 kDa. A Western Blot for Cdc34 was performed on the immunoprecipitates (Fig 6B α-Cdc34 immunoblot). Consistent with our earlier data, there appear to be novel Cdc34 interacting proteins as compared by the proteins of higher molecular weight in the Cdc34 immunoprecipitate.

DISCUSSION

Here we describe the partial purification and biochemical characterization of the Cdc34-associated protein complex in HeLa extracts required for DNA replication activity in Cdc34-immunodepleted *Xenopus* egg extracts. We show that anti-Cdc34 coupled beads incubated in a HeLa cell extract can rescue a Cdc34 immunodepleted *Xenopus* extract, which we call the Cdc34 associated DNA replication activity. Importantly, we have found that only a HeLa nuclear

extract that has been passaged over a phosphocellulose column exhibits the Cdc34 associated DNA replication activity (Fig 1). Further fractionation of the HeLa nuclear extract phosphocellulose flow-through by DEAE anion exchange, gel filtration and immunoaffinity chromatography found that only Cdc34 present in a large molecular size complex of ~400 kDa was able to rescue DNA replication in Cdc34-depleted *Xenopus* extract (Fig 2D). Immunoaffinity purification of the Cdc34 complex from partially purified HeLa nuclear extract indicated that SCF components, Cul1 and $p19^{Skp1}$, were not visibly associated with Cdc34, despite the ability of the Cdc34 complex to restore DNA replication to Cdc34-depleted *Xenopus* extract (Fig 5A,B). Further, the addition of recombinant human Cdc34 and $SCF^{p45Skp2}$ or another known Cdc34 interacting protein, CK2, was not sufficient to restore DNA replication to Cdc34-depleted *Xenopus* extract (Fig 5C). We cannot say by these studies, however, that the SCF/ring and or CK2 proteins are not necessary for complementation. These studies do suggest that the human Cdc34 complex does not consist merely of Cdc34 and SCF/ring components, but may instead consist of previously unidentified Cdc34-interacting proteins. Earlier purification studies in Xenopus indicate that only Cdc34 in a multi-protein complex of ~400 kDa was able to rescue a Xenopus Cdc34 immunodepleted egg extract [67]. Our studies in mammalian cells are consistent with these results and suggest the human and Xenopus protein complex may be conserved. It is unclear from the Xenopus studies if Cdc34 is associated with an inhibitor during any part of the cell cycle. The inhibition we observe in a nuclear extract could be another level of regulation for Cdc34 by direct or indirect means and are currently being investigated. We see a shift of Cdc34 in higher molecular weight fractions to that of lower molecular weight fractions in our sucrose gradients. In the HeLa NEXT the Cdc34 is found in higher molecular weight fractionates up to ~ 350 kDA by western analysis. This higher molecular weight form of Cdc34

shifts to ~ 180-225 kDa when the NEXT has been passaged over a phosphocellulose column (Fig 3). This shift suggests there may be a protein associated with the higher molecular weight Cdc34 complex that binds to the phosphocellulose column. We tried, unsuccessfully, to test these sedimentation fractions for DNA replication activity (data not shown). Importantly, we have found a purification step, HeLa PCFT/DEAE 150mM KCl bump, that appears to separate the bulk of Cdc34 that is not in a complex from a small percent of Cdc34 that is in a complex that rescues DNA replication activity in a Xenopus Cdc34 depleted egg extracts (Fig 4 A, C). When we immunopurify the HeLa PCFT/DEAE 150mM KCl bump on affinity purified Cdc34 beads we find candidate Cdc34 interacting proteins that do not appear to be known Cdc34 interacting proteins by molecular weight (Fig 6B). We have identified seven candidate Cdc34 interacting proteins with approximate molecular weights of; 56 kDa, 60 kDa, 90 kDa, 110 kDa, 120 kDa, 150 kDa, and 220 kDa. These molecular weights combined total a ~800 kDa which is greater than the ~400 kDa complex required for our Cdc34 rescuing activity. Identification of these proteins will help determine which ones are specific Cdc34 interacting proteins. In Xenopus, the functional Cdc34 that was able to rescue DNA replication was also found in a 440 KD complex. In addition, Xenopus extract (S100) immunoprecipitated with anti-Cdc34 coupled beads identified potential interacting proteins of Cdc34 corresponding to 60, 85, 180 and 220 kDa. The human immunopurified Cdc34 complex is sufficient for rescuing a Xenopus Cdc34 immunodepleted egg extract, suggesting this complex may be conserved between Xenopus and humans. The unidentified potential Cdc34 interacting proteins in mammalian cells appear to be similar (by molecular weight) to the candidate Cdc34 interacting proteins in Xenopus. Importantly, in both mammalian cells and in Xenopus, the associated Cdc34 proteins to not appear to be known SCF/ring proteins based on western analysis. A requirement for Cdc34 at

the initiation of DNA replication has been established. The substrates that must be degraded prior to the onset of DNA replication are still unknown. Identification of the Cdc34 interacting proteins will help elucidate the molecular mechanisms required for DNA replication initiation.

REFERENCES

FIGURE LEGENDS

Figure 1. *HeLa nuclear extract passaged over phosphocellulose can complement DNA replication in Cdc34-immunodepleted Xenopus egg extract.* Left panel (Depletion): Xenopus LSS was control depleted with pA-Seph coupled to normal rabbit serum Ig (Ctrl Depl), or pA-Seph coupled rabbit 1 anti-hCdc34 Ig (Cdc34 Depl) and sperm nuclei was added and assayed for DNA replication. Replication (%) is normalized to 100% of that in the control-depleted sample (Ctrl Depl). Right panel (Cdc34 Depletion/Rescue): 10uL of Cdc34 depleted Xenopus LSS left panel (Cdc34 Depl), was added to 8 uL pA-affiprep coupled to normal rabbit serum Ig (Ctrl) or pA-affiprep coupled anti-hCdc34 Ig incubated in LSS (XL LSS). Cdc34-depleted LSS (Cdc34 Depl) was also incubated with 8uL pA-affiprep coupled to anti-Cdc34 Ig and incubated with Hela nuclear extract (HeLa NEXT) or Hela nuclear extract passaged over a phosphocellulose column (HeLa PCFT). Rescuing Units were calculated by % replication of the rescue sample/% replication of control rescue.

Figure 2. *Cdc34 associated rescuing activity in mammalian cells is found in a high molecular weight complex.* **(A)** HeLa extract passaged over phosphocelluse (HeLa PCFT) was applied to a DEAE anion exchange column and eluted at increasing KCl bumps. The flow through (FT), 250 mM KCl bump (250), and 450mM KCl bump (450) fractions were subjected to western blot analysis with Cullin 1 (CUL 1), p45Skp2 (Skp2), affinity purified Cdc34 (Cdc34), p19Skp1 (Skp1) and Casein Kinase 2 (CK2). **(B)** The HeLa PCFT/DEAE 250mM KCl fraction was applied to SDEX 200 gel filtration chromatography. The molecular sizes listed above represent the elution of standards used to calibrate the column. Fractions were collected and subjected to western blot analysis with Cullin 1 (Cul1), p45Skp2 (Skp2), affinity purified Cdc34 (Cdc34) and

p19Skp1 (Skp1) or the starting material (HeLa PCFT/DEAE 250 mM KCl). **(C)** 3 μL of each SDEX200 fraction above were ran on a SDS-PAGE and silver stained. **(D)** Xenopus LSS was not depleted (No depl) or depleted with pA-Seph coupled to normal rabbit serum Ig (Ctrl), or pA-seph coupled rabbit 1 anti-hCdc34 Ig (Cdc34) and sperm nuclei was added and assayed for DNA replication. Replication (%) is normalized to 100% of that in the control-depleted sample (Ctrl). Right panel: 10uL of Cdc34 depleted LSS used in the left panel (Cdc34 Depl) was added to 8uL pA-affiprep coupled to normal rabbit serum Ig (Ctrl Rescue) or 8uL pA-affiprep coupled anti-hCdc34 Ig incubated in LSS. 10uL of Cdc34-depleted LSS (Cdc34 depl) was also incubated with 8 uL pA-affiprep coupled to anti-Cdc34 Ig incubated with HeLa PCFT/DEAE 250mM KCl fraction or SDEX 200 column fractions. Rescuing Units (RU) were calculated by % replication of the rescue sample/% replication of control rescue. The (+) represents the rescuing activity (Fig 4D fractions 10,11).

Figure 3. *Endogenous Cdc34 in mammalian cell extracts fractionated via sedimentation.* **(A)** HeLa whole cell extract (HeLa WCE) was layered on an isokinetic 10-40% sucrose gradient in parallel with protein standards. Fractions were collected from the top of the gradient (10%) followed by western blot analysis with Casein Kinase 2 antibody (ANTI-CK2), affinity purified Cdc34 antibody (ANTI-Cdc34) or p19Skp1 antibody (ANTI-p19Skp1). Molecular weights are shown above the panel. **(B)** HeLa Phosphocellulose flow through (HeLa PCFT) or HeLa nuclear extract (HeLa NEXT) were layered on an isokinetic 10-40% sucrose gradient as just described. Fractions were collected from the top of the gradient (10%) followed by western blot analysis with affinity purified Cdc34 antibody (ANTI-Cdc34). Molecular weights are shown above the panel.

Figure 4. *Fractionation of HeLa Phosphocellulose Flow Through over a DEAE column and bumping at 150 mM KCl seperates the small percent of Cdc34 in an active complex.* **(A)** HeLa extract passaged over phosphocelluse (HeLa PCFT) was applied to a DEAE column and eluted at increasing KCL bumps. The flow through (FT), 150 mM KCl, 300mM KCl, and 600mM KCl bump fractions were subjected to western blot analysis with Cullin 1 (CUL 1), p45Skp2 (Skp2), affinity purified Cdc34 (Cdc34), p19Skp1 (Skp1). **(B)** 3 µL of each DEAE fraction above were ran on a SDS-PAGE and silver stained. **(C)** Left panel: Xenopus LSS was not depleted (No depl) or depleted with pA-Seph coupled to normal rabbit Ig (Ctrl), or pA-seph coupled rabbit 1 anti-hCdc34 Ig (Cdc34) and sperm nuclei was added and assayed for DNA replication activity. Replication (%) is normalized to 100% of that in the control-depleted sample (Ctrl). Right panel: 10uL Cdc34 depleted LSS used in the left panel (Cdc34 Depl) was added to pA-affiprep coupled to normal rabbit Ig (Ctrl) or pA-affiprep coupled anti-hCdc34 Ig incubated in LSS. Cdc34-depleted LSS (Cdc34 depl) was also incubated with pA-afffiprep coupled to anti-Cdc34 Ig and incubated with Hela PCFT/DEAE Flow Through (FT) fraction, 150mM KCl bump, 300mM KCl bump, and 600mM KCl bump. Rescuing Units were calculated by % replication of the rescue sample/% replication of control rescue. The astric (*) represents rescuing activity (Fig 4C).

Figure 5. *Known Cdc34 interacting proteins do not immunoprecipitate with Cdc34 nor rescue a Cdc34 depleted Xenopus egg extract.* **(A)** 2mgs of HeLa phosphocellulose flow through (HeLa PCFT) were incubated with pA-affiprep coupled to anti-Cdc34 Ig (anti-Cdc34) or to pA-affiprep coupled to normal rabbit serum Ig (Ctrl) and washed. Sample buffer was added to the beads and boiled. The eluent or 50ug of HeLa PCFT or HeLa PCFT/DEAE 250mM KCl fraction.was

subjected to western analysis with Cullin 1 (Cul1), affinity purified Cdc34 (Cdc34) or p19Skp1 (Skp1) **(B)** Left panel: Xenopus LSS was not depleted (No depl) or depleted with pA-Seph coupled to preimmune Ig (Ctrl), or rabbit 1 anti-hCdc34 Ig (Cdc34) and sperm nuclei was added. Replication (%) is normalized to 100% of that in the control-depleted sample (Ctrl). Right panel: Cdc34 depleted LSS used in the left panel (Cdc34 Depl) was added to pA-affiprep coupled to preimmune Ig (Ctrl Rescue) or anti-hCdc34 Ig incubated in LSS. 200nM Cdc34p with 200nM Cul1/ROC1, 200nM Cdc34p with Cul1/ROC1/Skp1/Skp2 or 200nM Cdc34p with 200nM Casein Kinase 2 (CK2) was added to the Cdc34 depleted LSS and examined for Cdc34 rescuing activity. Rescuing Units were calculated by % replication of the rescue sample/% replication of control rescue.

Figure 6. *Fractionation schematic of Cdc34 rescuing activity in mammalian cell extracts.* **(A)** Fractionation scheme. HeLa cell extracts were fractionated and subjected to Cdc34 rescuing assays in Xenopus Cdc34 depleted extracts. For details see Results. **(B)** Silver Stain SDS-PAGE. Top Panel: (Silver Stain): M indicate molecular weight markers in kDa. .1% of the immunoprecipitation input of the HeLa PCFT/DEAE 150mM KCl fraction (.1% Input). HeLa PCFT/DEAE 150mM KCl fraction was immunoprecipitated with affinity purified anti-Cdc34 (Cdc34 IP) or RIgG (RigG IP) and eluted by acid base. The eluent was concentrated and resolved by SDS-PAGE and silver stained as described in the methods. Bottom Panel (Western). $1/5^{th}$ of the concentrated eluent from each IP (Top panel) was resolved on a SDS-Page gel, transferred to nitrocellulose and examined by western blot for Cdc34 (α-Cdc34 immunoblot.

Figure 1

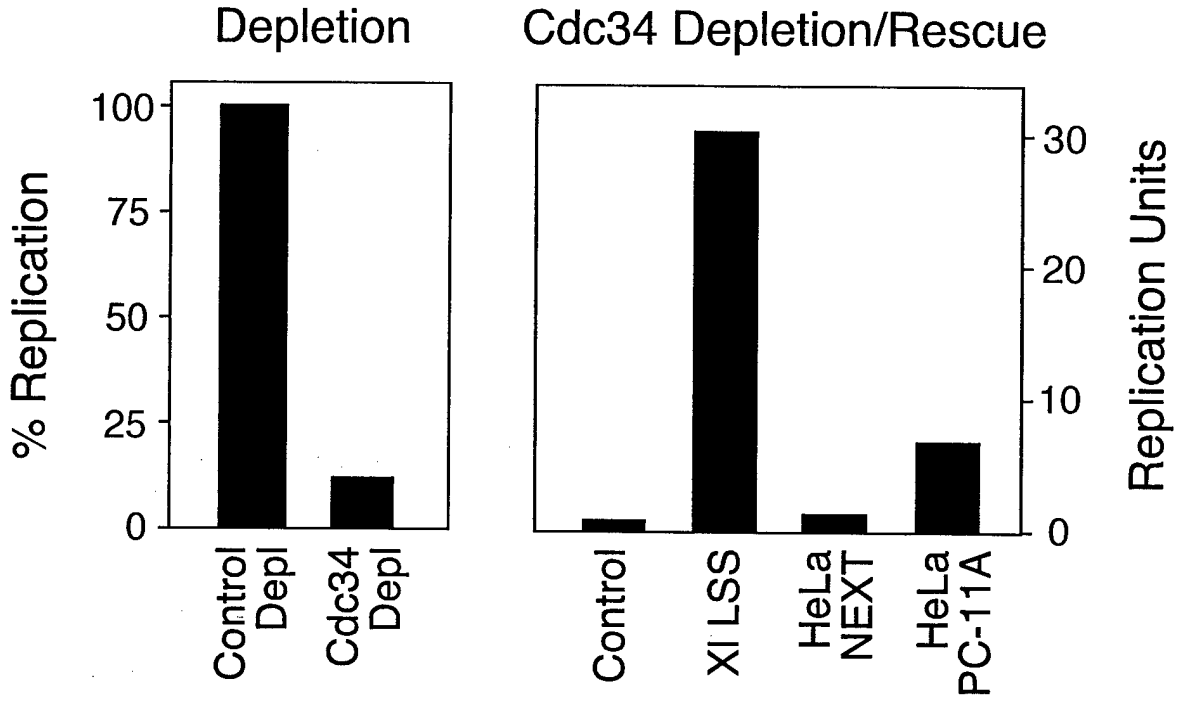

Depletion
Cdc34 Depletion/Rescue
% Replication
100
75
50
25
0
Control Depl
Cdc34 Depl
30
20
10
0
Replication Units
Control
XI LSS
HeLa NEXT
HeLa PC-11A

Figure 2

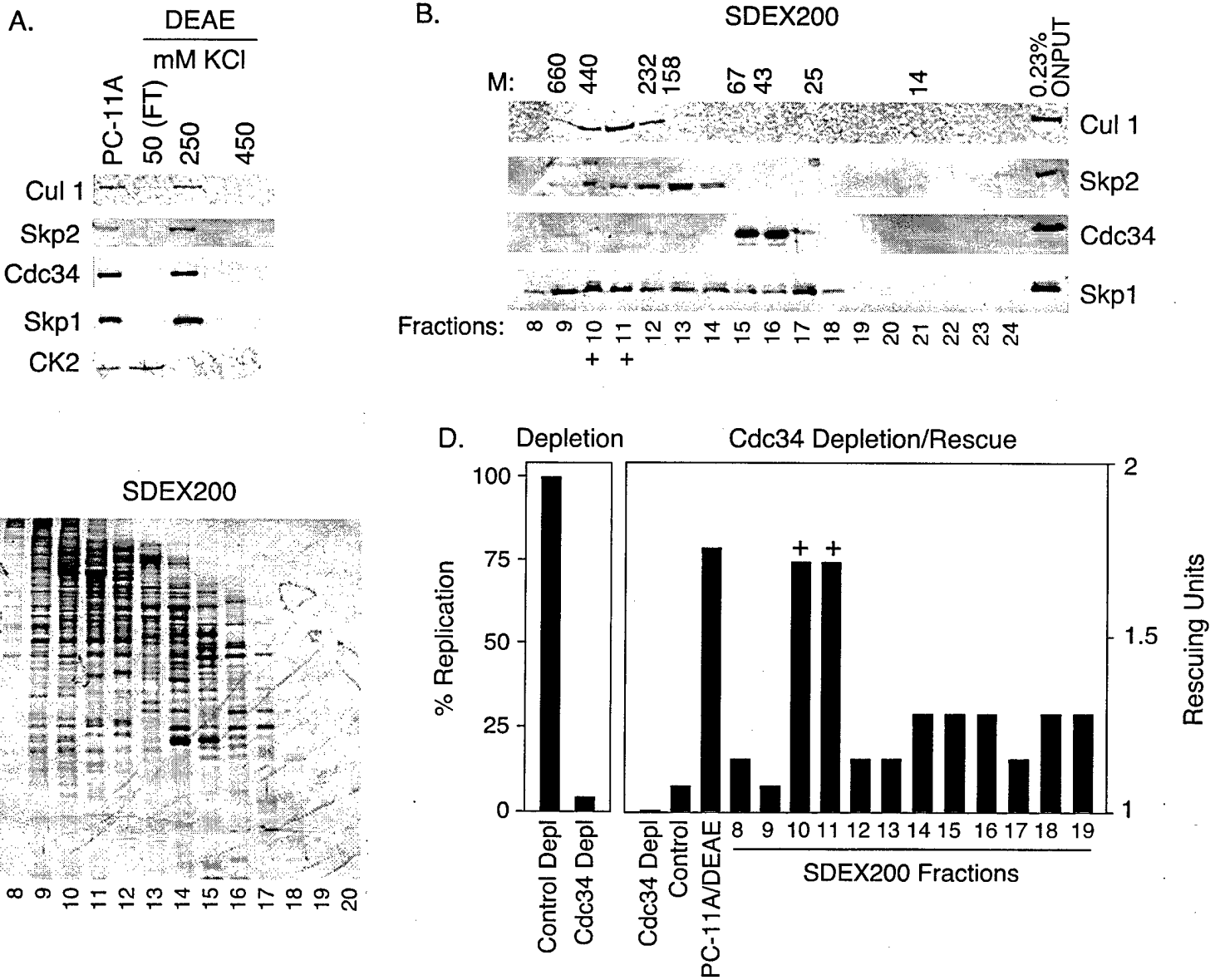

A.
DEAE
mM KCl
PC-11A
50 (FT)
250
450
Cul 1
Skp2
Cdc34
Skp1
CK2
B.
SDEX200
M:
660
440
232
158
67
43
25
14
0.23% INPUT
Cul 1
Skp2
Cdc34
Skp1
Fractions:
8 9 + 10 + 11 12 13 14 15 16 17 18 19 20 21 22 23 24
C.
SDEX200
M
190
120
85
60
50
40
25
20
15
Fractions:
8 9 10 11 12 13 14 15 16 17 18 19 20
D.
Depletion
Cdc34 Depletion/Rescue
% Replication
100
75
50
25
0
Rescuing Units
2
1.5
1
+
+
Control Depl
Cdc34 Depl
Cdc34 Depl
Control
PC-11A/DEAE
8 9 10 11 12 13 14 15 16 17 18 19
SDEX200 Fractions

Figure 3

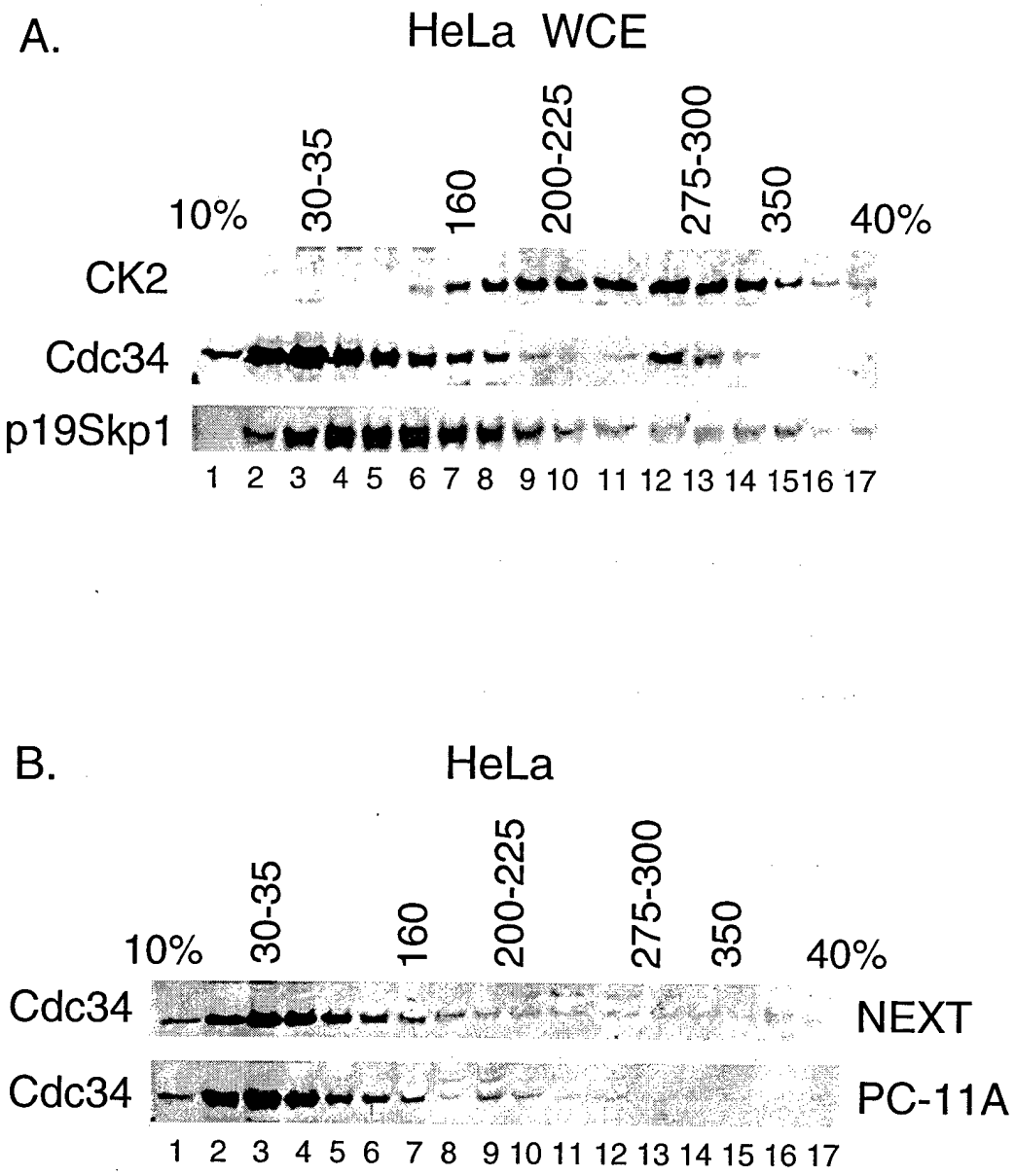

A.
HeLa WCE
10%
30-35
160
200-225
275-300
350
40%
CK2
Cdc34
p19Skp1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
B.
HeLa
10%
30-35
160
200-225
275-300
350
40%
Cdc34
NEXT
Cdc34
PC-11A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Figure 4

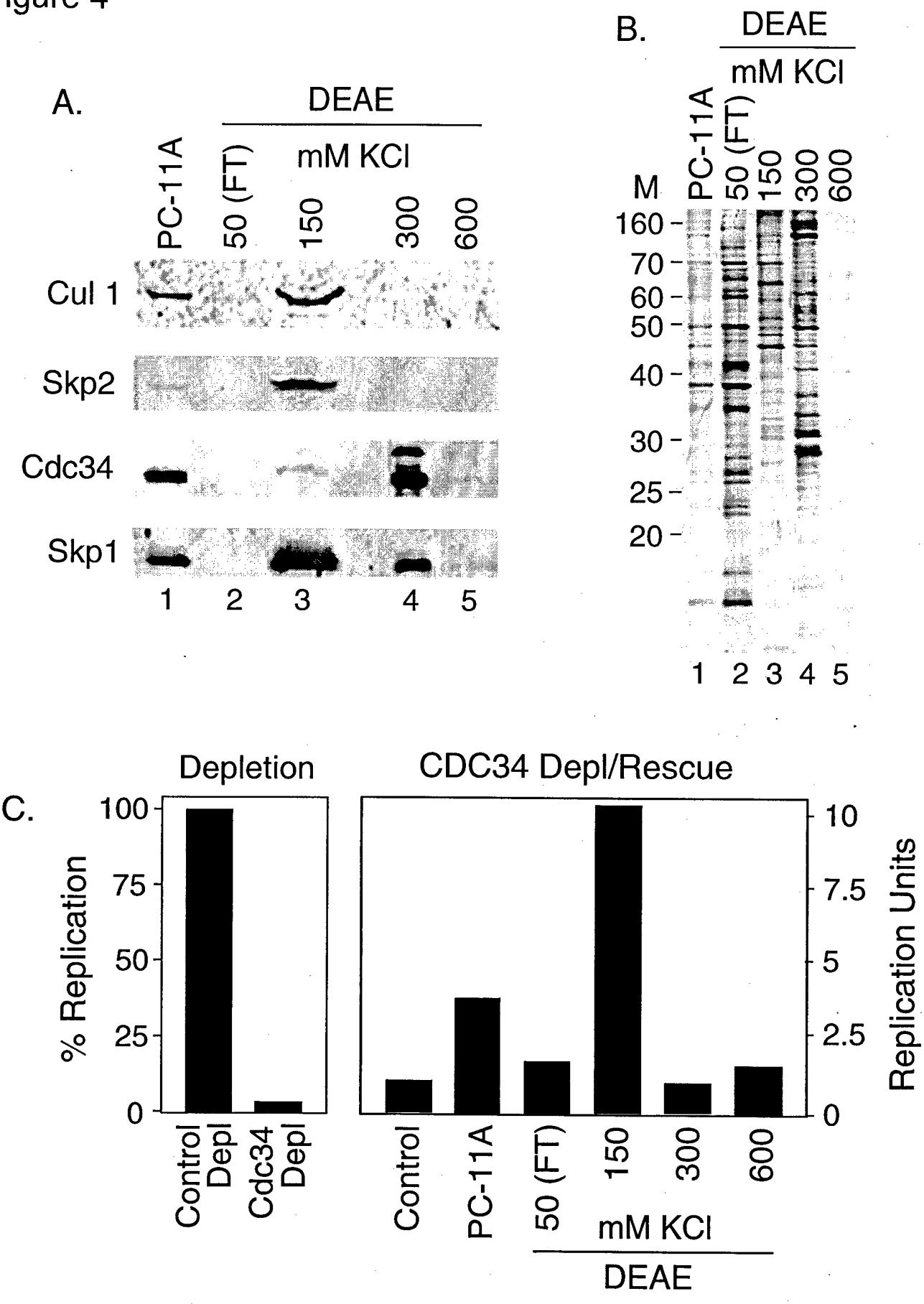
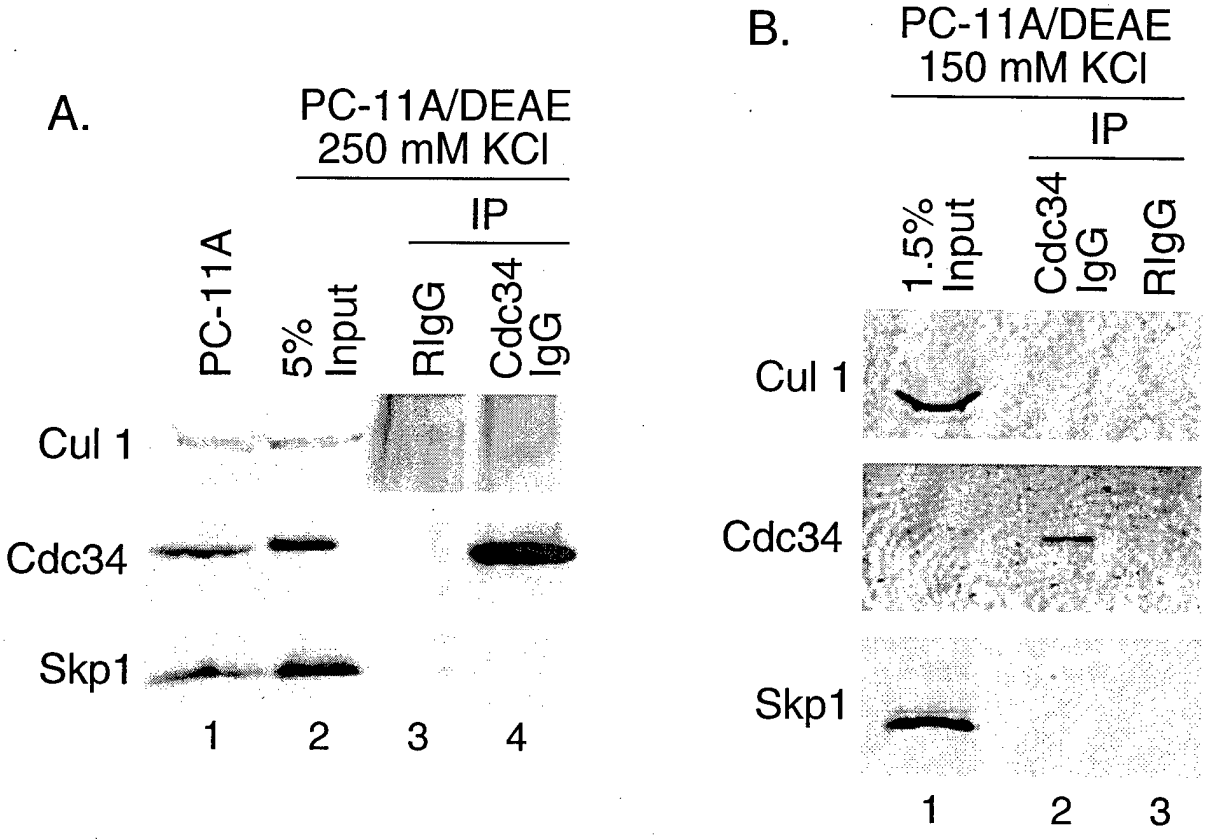

A.
DEAE
PC-11A
50 (FT)
mM KCl
150
300
600
Cul 1
Skp2
Cdc34
Skp1
1 2 3 4 5
B.
DEAE
mM KCl
M
PC-11A
50 (FT)
150
300
600
160
70
60
50
40
30
25
20
1 2 3 4 5
C.
Depletion
CDC34 Depl/Rescue
% Replication
100
75
50
25
0
Control Depl
Cdc34 Depl
Replication Units
10
7.5
5
2.5
0
Control
PC-11A
50 (FT)
150
300
600
mM KCl
DEAE

Figure 5


A.
PC-11A/DEAE
250 mM KCl
IP
PC-11A
5% Input
RIgG
Cdc34 IgG
Cul 1
Cdc34
Skp1
1
2
3
4
B.
PC-11A/DEAE
150 mM KCl
IP
1.5% Input
Cdc34 IgG
RIgG
Cul 1
Cdc34
Skp1
1
2
3

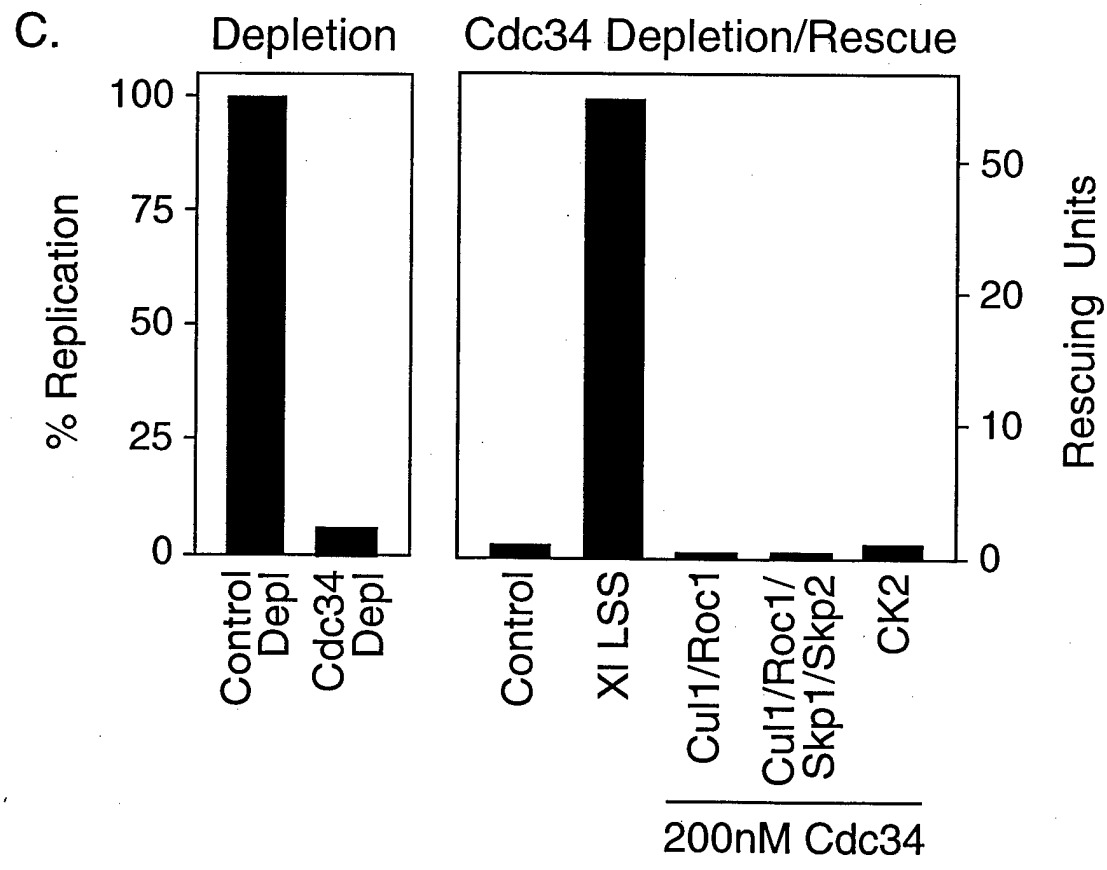
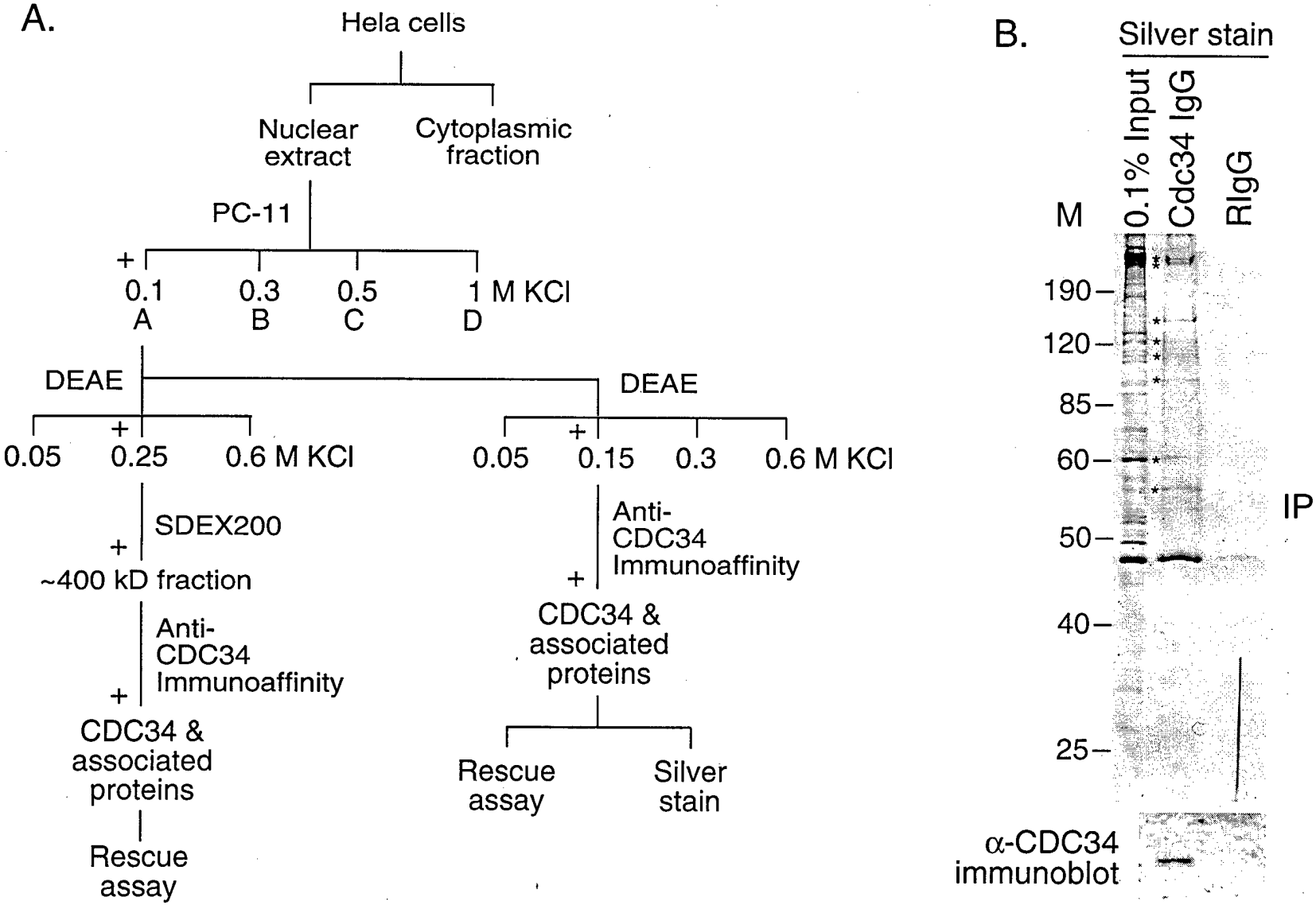

C.
Depletion
Cdc34 Depletion/Rescue
% Replication
100
75
50
25
0
Rescuing Units
50
20
10
0
Control Depl
Cdc34 Depl
Control
XI LSS
Cul1/Roc1
Cul1/Roc1/ Skp1/Skp2
CK2
200nM Cdc34

Figure 6


A.
Hela cells
Nuclear extract
Cytoplasmic fraction
PC-11
+
0.1 A
0.3 B
0.5 C
1 M KCl D
DEAE
DEAE
+
0.05
0.25
0.6 M KCl
0.05
0.15
0.3
0.6 M KCl
SDEX200
+
~400 kD fraction
Anti-CDC34 Immunoaffinity
+
CDC34 & associated proteins
Rescue assay
Anti-CDC34 Immunoaffinity
+
CDC34 & associated proteins
Rescue assay
Silver stain
B.
Silver stain
M
0.1% Input
Cdc34 IgG
RIgG
190
120
85
60
50
40
25
IP
α-CDC34 immunoblot